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**Prostate Cancer Evaluation: Design, Synthesis and Evaluation of Novel Enzyme-Activated Proton MRI Contrast Agents**


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**14. ABSTRACT**

The lacZ gene encoding E. coli beta-gal has already been recognized as the most commonly used reporter system in cancer gene therapy. Moreover, prostate-specific membrane antigen (PSMA) has been identified as an ideal antigenic target in prostate cancer. We propose to develop a novel class of Gd(III)-based MRI contrast agents for in vivo detection of beta-gal or PSMA activity. This new concept of the Gd(III)-based MRI contrast agents is composed of three moieties: (A) a signal enhancement group, such as Gd-DOTA or Gd-PCTA; (B) an Fe(III) chelating group; (C) beta-D-galactose or glutamate. Following cleavage by lacZ transgene or PSMA in prostate cancer cells, the released, activated aglycone Fe(III)-ligand will spontaneously trap endogenous Fe(III) at the site of enzyme activity forming a highly stable complex, to restrict motion of the Gd(III) chelates enhancing relaxivity and providing local contrast accumulation. We plan to synthesize 8 novel MRI contrast agnets for imaging beta-gal or PSMA activity in prostate cancer cell culture, explore the feasibility of applying the most promising analogies to cells grown in vivo in mice and rats.

**15. SUBJECT TERMS**

Prostate Cancer Evaluation, Contrast Agent, Synthesis, MRI, Gene Expression, Gene Therapy, in vivo Cancer Imaging, lacZ Gene, beta-Galactosidase, PSMA, NAALADase

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**Prostate Cancer Evaluation: Design, Synthesis and Evaluation of Novel Enzyme-Activated ^{1}H MRI Contrast Agents**

## INTRODUCTION

**BACKGROUND** Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men in the United States, in 2009, approximately 192,000 men were diagnosed with prostate cancer with 27,000 succumbing to this disease,[1,2] currently, there is no cure for locally advanced or metastatic prostate cancer.

Gene therapy has emerged as a potentially promising strategy for treatment of prostate cancer.[3-15] The prostate is particularly amenable to gene therapy.[11-16] However, there are major issues in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed.[15-33] A viral construct is often readministered on successive occasions, but this should optimally be timed to coincide with loss of expression. Inevitably gene therapy has associated risks, and thus non-invasive *in vivo* determining the duration of gene expression in an individual tumor could greatly enhance the viability of the approach. Gene expression now is commonly monitored by *in situ* hybridization techniques or by introducing a marker gene to follow the regulation of a gene of interest. Since β-galactosidase (β-gal) activity is readily assessed by histology or in culture, in hosts as evolutionarily diverse as bacteria, yeast, and mammals, its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction, *lacZ* gene encoding *E. coli* β-gal has already been recognized as the most commonly used reporter system.[34] A variety of *lacZ* gene reporters has been developed, such as colorimetric,[35-39] fluorescence,[40-53] chemiluminescence,[54-61] radiotracers for positron emission tomography (PET) or single-photon emission computed tomography (SPECT),[62-66] magnetic resonance imaging (MRI) probes,[67-69] and ^{19}F-NMR approaches,[70-77] though most of them have only been utilized in *in vitro* detection, with a very few successful applications *in vivo* so far.[39,49,50,51,60,63-65,67,68,76,77] Therefore, the development of non-invasive *lacZ* gene reporter techniques based on appropriate molecules and imaging modalities is still a high desire.

The superb spatial resolution and the outstanding capacity of differentiating soft tissues have determined the widespread success of magnetic resonance imaging (MRI) in clinical diagnosis.[78] The contrast in an MR image is the result of a complex interplay of numerous factors, including the relative $T_1$ and $T_2$ relaxation times, proton density of the imaged tissues and instrumental parameters. It was shown that contrast agent causes a dramatic variation of the water proton relaxation rates, thus providing physiological information beyond the impressive anatomical resolution commonly obtained in the uncontrasted images. Contrast agents are widely used clinically to assess organ perfusion, disruption of the blood-brain barrier, occurrence of abnormalities in kidney clearance, and circulation issues.[78-82] The responsive MRI contrast agents holds great promise in the gene therapy arena.[83,84] The abilities of these contrast agents to relax water protons is triggered or enhanced greatly by recognition of a particular biomolecule opening up the possibility of developing MRI tests specific for biomarkers indicative of particular disease states and aiding in the early detection and diagnosis of tumors. Desreux *et al* [80,85] demonstrated that, by chelating Gd(phen)HDO3A with Fe(II) to form a highly stable tris-complex, as shown in **Figure 1**, the relaxivity increased 145% at 20MHz and 37°C from 5.1mM$^{-1}$s$^{-1}$ per Gd(III) in Gd(phen)HDO3A form to 12.2 mM$^{-1}$s$^{-1}$ in the tris-complex. Desreux *et al* [80,85] also synthesized another iron-sensitive MRI contrast agent with a tris-hydroxamate (**Figure 2**). After the tris-

hydroxamate groups formed a chelate with Fe(III), free rotation at the Gd(III) centers was restricted, thereby increasing relaxivity by 57% from 5.4 to 8.5mM$^{-1}$s$^{-1}$ at 20 MHz.
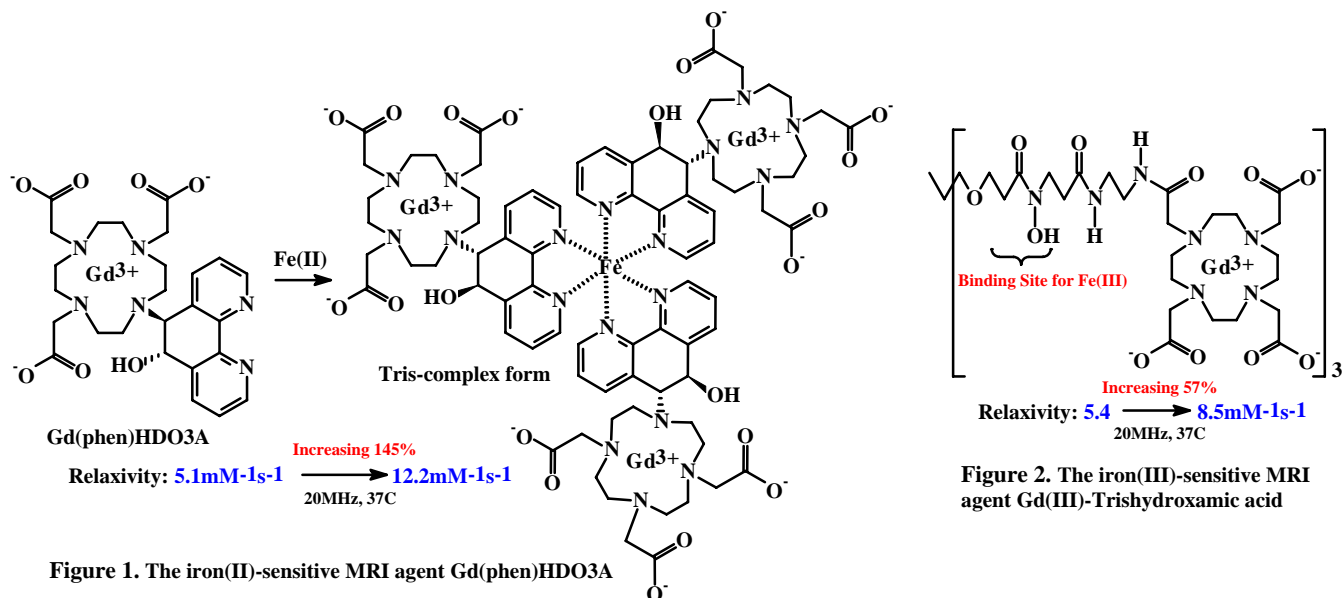
**Figure 1. The iron(II)-sensitive MRI agent Gd(phen)HDO3A**

**Figure 2. The iron(III)-sensitive MRI agent Gd(III)-Trishydroxamic acid**

Most recently, Merbach *et al* [86-88] observed the remarkably high $T_1$ relaxivity gain by the heterometallic, self-assembled metallostar formation with six efficiently relaxing Gd$^{III}$ centers from (tpy-DTTA)Gd(H$_2$O) with 7.3mM$^{-1}$s$^{-1}$ to {Fe$^{II}$[Gd$^{III}$$_2$(tpy-DTTA)$_2$(H$_2$O)$_4$]$_3$}$^{4-}$ with 15.7mM$^{-1}$s$^{-1}$ at 20MHz and 37°C (**Figures 3**), significantly, their detailed studies on structure and dynamics of the trinuclear complex {Fe$^{II}$[Gd$^{III}$$_2$(tpy-DTTA)$_2$(H$_2$O)$_4$]$_3$}$^{4-}$ indicate that the heterometallic self-assemblies attain high $T_1$ relaxivities by influencing three factors: water exchange, rotation, and electron relaxation.
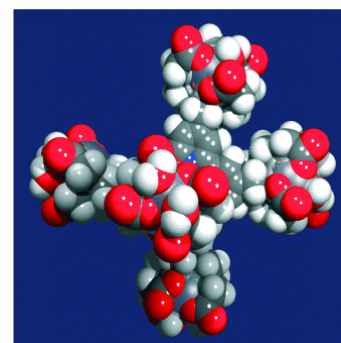
**Figure 3.** {Fe[Gd$_2$L$_2$(H$_2$O)$_4$]$_3$}$^{4-}$

**DESIGN**  Prompted by these findings, we proposed a novel class of enzyme responsive Gd$^{3+}$-based MRI contrast agent for high sensitivity and specificity for β-gal detection, based on the tumor biology and Fe-chelation therapeutic strategy. Cancer cells, as compared with their normal counterparts, exhibit increased uptake and utilization of more iron, as evidenced by an increase in transferrin receptors at the cancer cell surface, mediating a high level and rate of iron uptake.[89] More recently, an emerging class of Fe-chelator agents have shown effective antitumor activity *in vitro* and *in vivo*, which can overcome resistance to standard chemotherapy, due to their ability to affect multiple molecular targets including the enzyme responsible for the rate-limiting step of DNA synthesis, ribonucleotide reductase, molecules involved in cell cycle control (*e.g.* cyclin D1, p21$^{CIP1/WAF1}$) and the inhibition of metastasis (*i.e.* N-myc downstream regulated gene-1).[89-94] The FDA has approved five Fe-chelators for use in anticancer therapy so far, some others are in clinical trials for the treatment of various metastatic and solid cancers.[89,95-97] In our design, the *lacZ* responsive Gd$^{3+}$-based MRI contrast agent is comprised of three moieties: (A) a signal enhancement group, such as Gd-DOTA or Gd-PCTA; (B) an Fe$^{3+}$ chelating group; (C) β-D-galactose. Upon encountering with β-gal in tumor cells, the released, activated Fe$^{3+}$-ligand will spontaneously scavenge tumor abundant Fe$^{3+}$ at the site of enzyme activity forming a highly

stable Fe-complex, to localize and accumulate the signal enhancement groups (*e.g.* Gd-DOTA or Gd-PCTA) in tumor, revealing regional β-gluc activity, and verifying the location and magnitude of tumor to evaluate the gene therapy. Also, the formation of the Fe-complex will restrict motion of the $Gd^{3+}$ chelates, then enhancing additional relaxivity. **Figure 4** depicts the mechanism for detection of *lacZ* gene expression through $Fe^{3+}$-trapped MRI contrast agent formation.
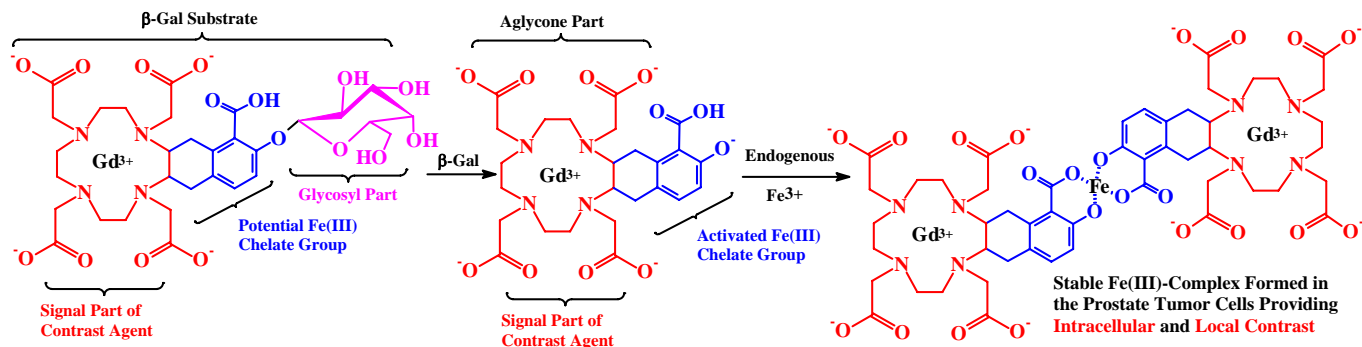


**Figure 4.** Detection of *lacZ* gene expression by β-gal activated *in situ* $Fe^{3+}$-trapped MRI contrast agent formation.

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein with enzymatic activities: N-acetylated α-linked L-amino dipeptidase (NAALADase) and γ-glutamyl carboxypeptidase (folate hydrolase).[97-99] Studies with the monoclonal antibodies have demonstrated that PSMA is the most well-established, highly restricted prostate cancer cell surface antigen, it is expressed at high density on the cell membrane of all prostate cancers.[100-102] The high prostate tissue specificity of PSMA has been identified as an ideal therapeutic and diagnostic target for prostate cancer, this potential was exemplified by the recent FDA approval of an $^{111}$In-labeled PSMA monoclonal antibody (Prostascint**®**) for diagnostic imaging of prostate cancer.[103-110] Furthermore, phase I and II trials have begun using immunotherapy directed against PSMA.[106-108] By introducing γ-glutamate residue instead of D-galactose in the Figure 4, we intend to develop a novel class of PSMA responsive Gd(III)-based MRI approach specific for prostate cancers detection with high sensitivity (**Figure 5**).
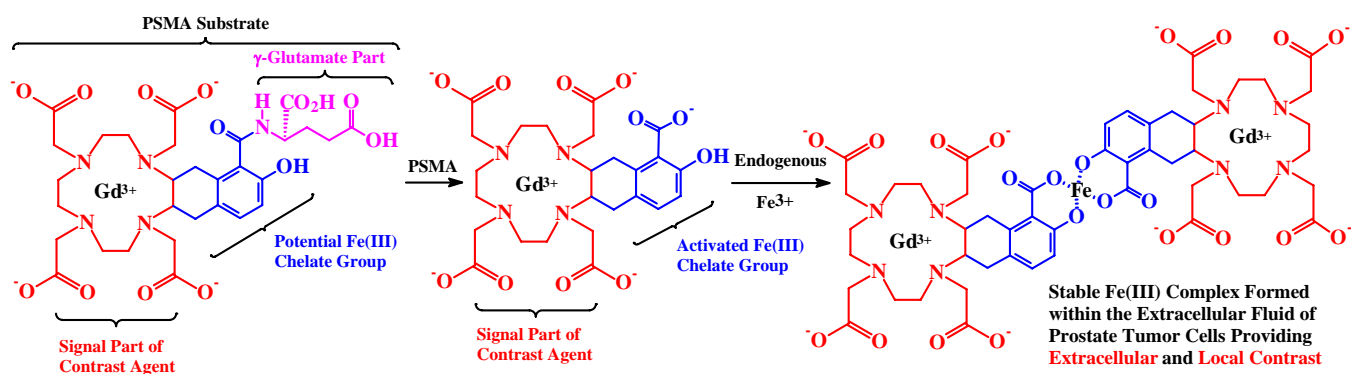


**Figure 5.** PSMA responsive Gd(III)-based MRI approach specific for prostate cancers detection.

Especially, PSMA has a large extracellular domain,[108] so the expression of PSMA tethered to the surface of the prostate cancer cells makes that the above novel peptide-based MRI contrast agents can be activated extracellularly around prostate cancers,[109] thus the need for a peptide-based MRI contrast agent to penetrate the prostate tumor cell membrane is no longer a prerequisite. The

permeability is always one of the greatest challenges in the development of *in vivo* MRI contrast agents.[111]

Accordingly, depending upon the enzyme sources either being the *lacZ* transgene or the PSMA from prostate tumors, this new platform could provide *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular** contrast agents), with combining the factors of reaching the high relaxivities. Furthermore, this new class of responsive MRI contrast agent is composed of three functional moieties, in which the signal enhancing and $Fe^{3+}$ chelating parts are changeable allowing modification in a search for ideal $Fe^{3+}$-trapped MRI contrast agents. Importantly, the combination of three functional moieties is based on the clinically applied strategies on cancer therapy. These facts strongly suggest the potential of the proposal to future clinical application.

## STATEMENT OF WORK

**Specific Aim 1** Design and synthesize model "smart" MRI contrast agents to report β-gal or PSMA activities with the ability of trapping $Fe^{3+}$ ion.

**Task 1** Design and optimization of synthetic strategies for reporter molecules. (**Completed**)

**Task 2** Structural characterizations of the synthesized molecules. (**Completed**)

**Specific Aim 2** Test the properties of molecules in solution and *in vitro* with cultured prostate cancer cells.

**Task 3** Evaluation the basic properties of the agents **in solution**. (**Completed**)

**Task 4** Evaluation of the properties of the optimal molecules *in vitro* **with cultured prostate cancer cells**. (**Completed**)

**Specific Aim 3** Scale up synthesis of the most promising MRI contrast agent(s) and apply to animal investigations.

**Task 5** Scale up synthesis of the most promising $^1$H MRI contrast agent(s). (**Completed**)

**Task 6** Apply the most promising $^1$H MRI contrast agent(s) to assess β-gal transfection efficiency, *lacZ* gene expression (spatial and temporal) in prostate tumors *in vivo*. (**Completed**)

**Task 7** Test dosing protocols, timing, MR detection protocols (**Completed**)

**Task 8** Prepare manuscripts and final report (**Completed**)

## BODY

**SEYNTHESIS** Initially, we started the syntheses of the target molecules with the strategy of constructing the structures of $Gd^{3+}$ and $Fe^{3+}$ chelators simultaneously in the fused way as designed in the proposal, in order to maximize the restriction for the motion of the $Gd^{3+}$ chelates, then obtaining the optimal relaxivity.

In the years 1 and 2, the syntheses according to the original plan met the challenges on (1) selective removal of benzyl ether (in blue) in the presence of benzyl ester (in magenta) by Pd/C hydrogenolysis; (2) selective removal of esters to accomplish the expected compound **D**, then to the target molecule **M$_1$** (see **Figure 6**). Because **M$_3$~M$_6$** are analogues of **M$_1$** and **M$_2$**, similarly, their syntheses had encountered the same situations. Although we put in much time and effort for solving these issues even partially in year 3, the failure made us modify the synthetic strategy by using tert-Butyl (instead of Ethyl) (see **Figure 7**), since they can be readily and selectively removed.
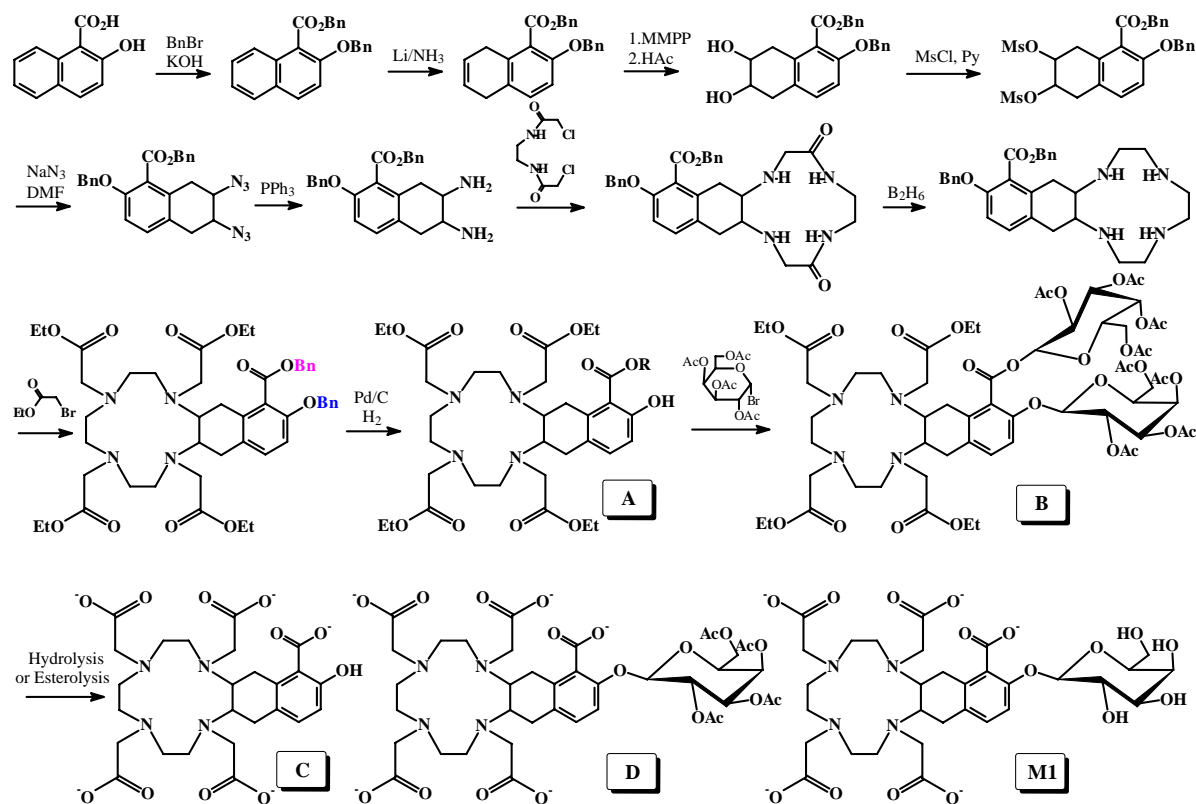
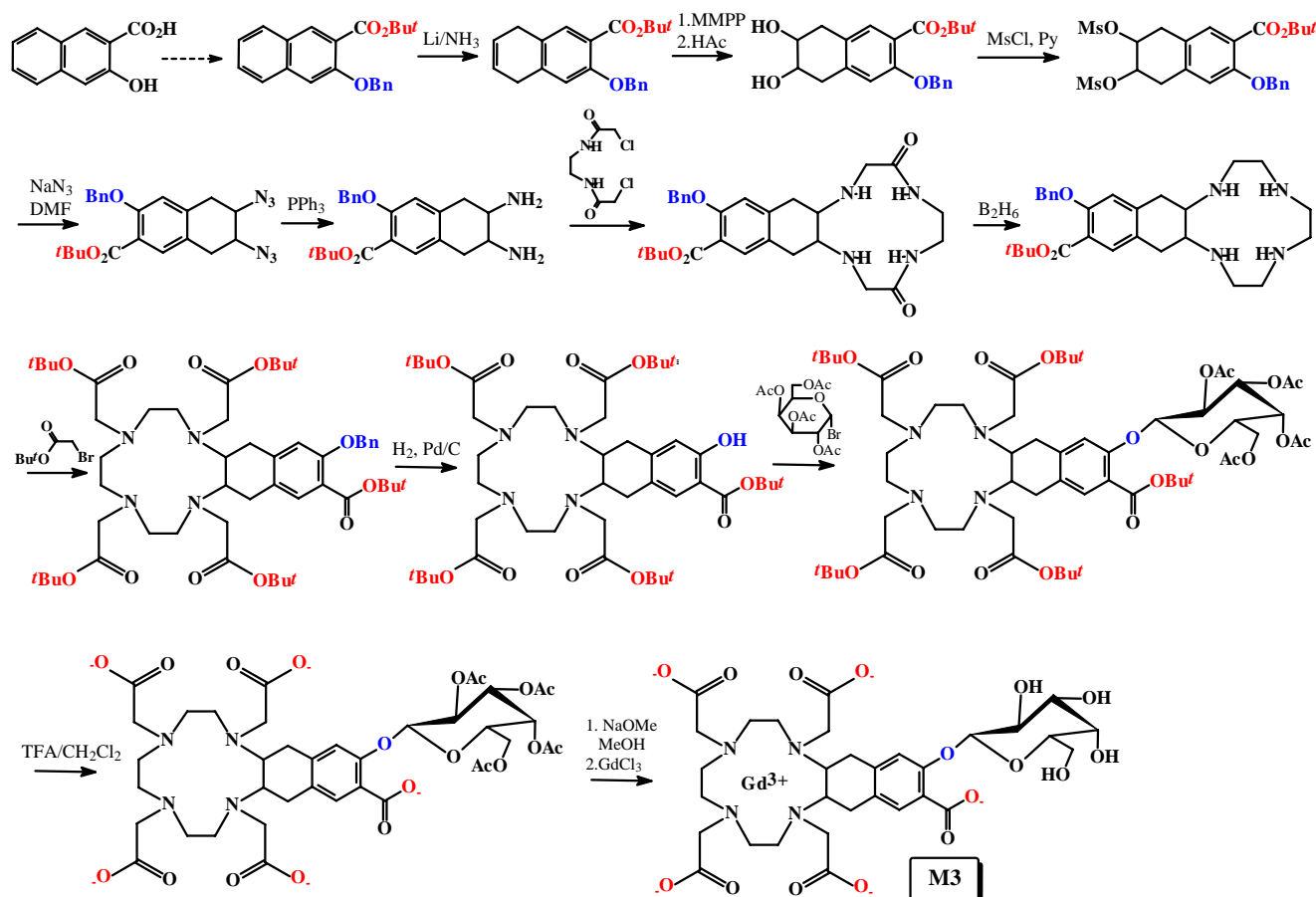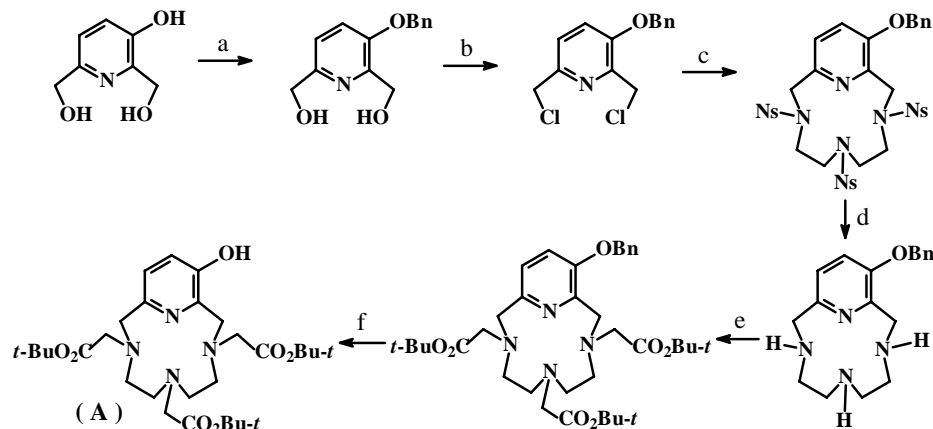**Figure 6.** Initial Synthetic Strategies for $M_1 \sim M_6$.



**Figure 7.** Modified Synthetic Strategies for $M_1 \sim M_6$.

Following the modified synthetic strategy, we eventually achieved the designed target molecules **M₁~M₆** and **M₇**, **M₈** (see **Figure 8**).



**Reaction Conditions:** (a) BnBr, K$_2$CO$_3$, MeCN, 79%; (b) Bu$_3$P, CCl$_4$, 80%; (c) NsNH(CH$_2$)$_2$N(Ns)(CH$_2$)$_2$NHNs, K$_2$CO$_3$, MeCN, 81%; (d) HSCH$_2$COOH, LiOH, DMF, 75%; (e) BrCH$_2$CO$_2$Bu-$t$, K$_2$CO$_3$, 85%; (f) H$_2$, Pd/C, 90%.
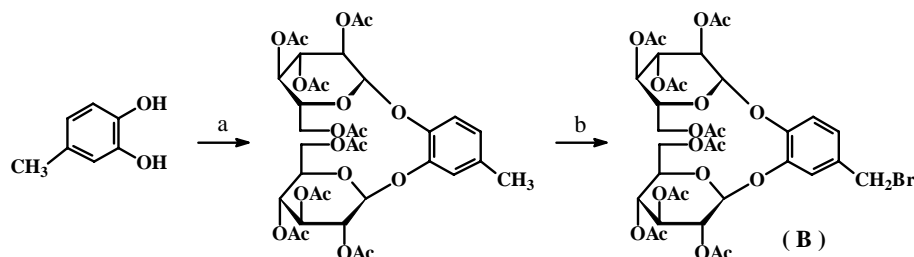


**Reaction Conditions:** (a) 2,3,4,6-tetra-$O$-acetyl-$a$-D-galactopyranosyl bromide, Hg(CN)$_2$, 4Å M.S., CH$_2$Cl$_2$, 88%; (b) NBS, 78%;
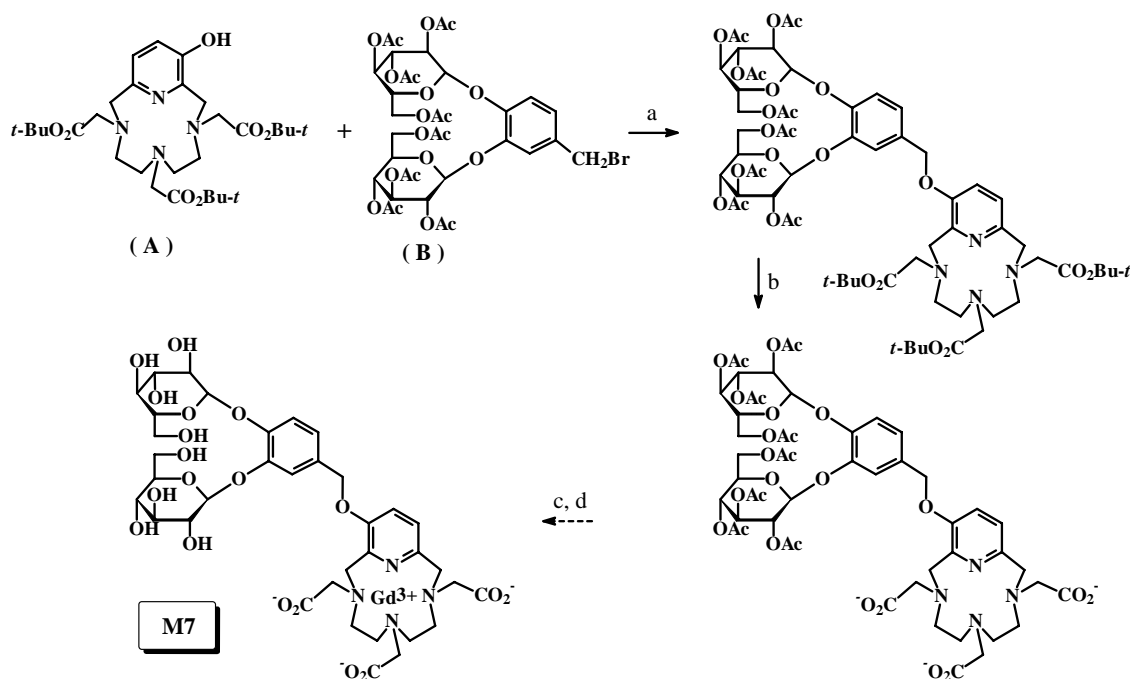


**Figure 8. Reaction Conditions:** (a) K$_2$CO$_3$, MeCN, 75%; (b) CF$_3$CO$_2$H, CH$_2$Cl$_2$, 81%; (c) GdCl$_3$, Pyridine, 82%; (d) MeOH, MeONa, 89%.

The test of the target molecules **M$_1$~M$_8$** using [1]H-MRI by comparing the contrast enhancement with that of the control in sodium phosphate buffer solution (PBS) (0.1 M, pH=7.4) in the presence of ferric ammonia citrate (FAC) with β-galactosidase E801A or PSMA (from lysed LNCaP cells in Tris buffer) indicated that: (1) the reporter molecules **M$_1$, M$_3$, M$_5$, M$_7$** can not be hydrolyzed by β-galactosidase E801A; (2) the reporter molecules **M$_2$, M$_4$, M$_6$, M$_8$** cannot be hydrolyzed by PSMA; so no MRI contrast changes before and after addition of β-galactosidase E801A or PSMA can be seen (**Figure 9** and **Table 1**).
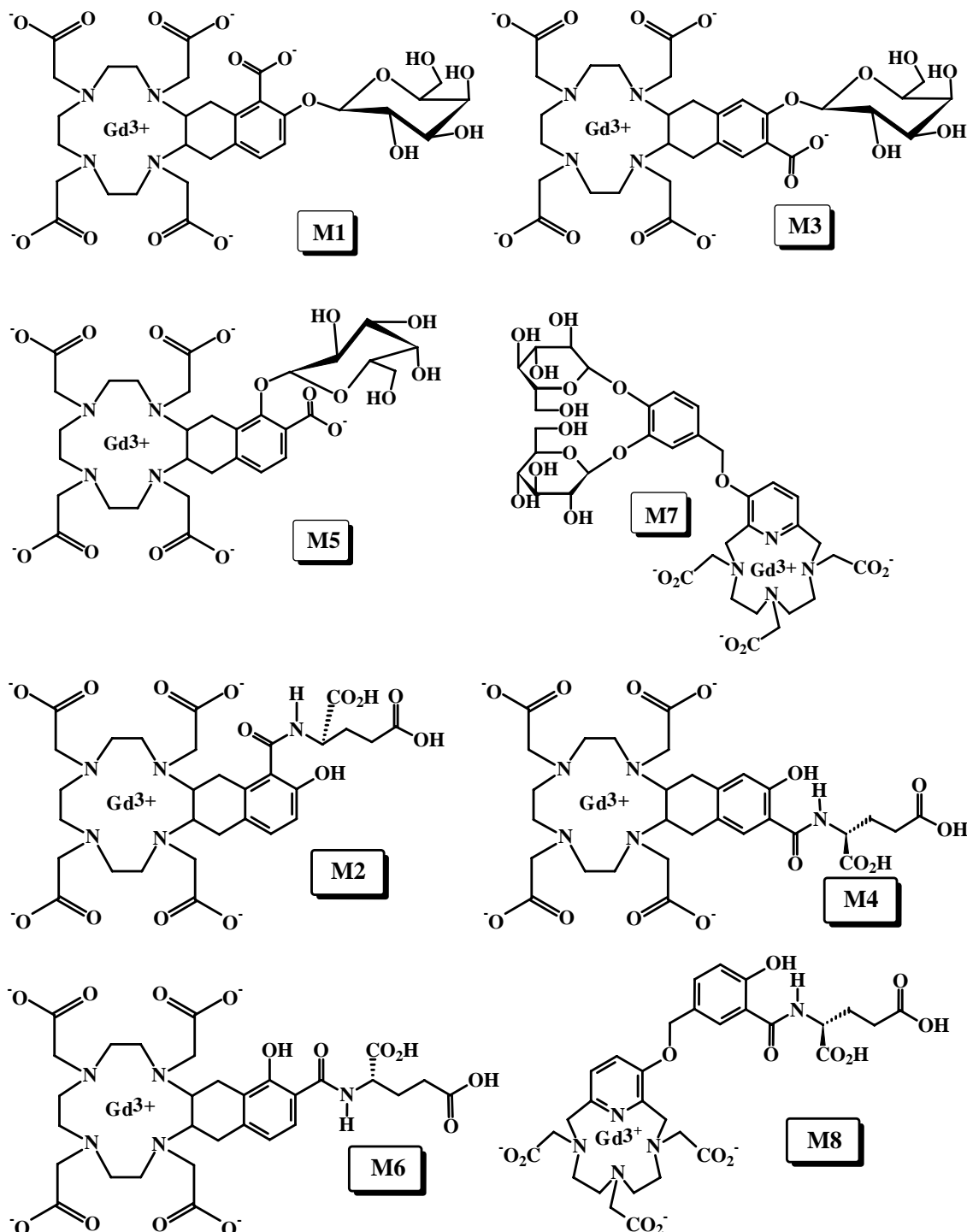


**Figure 9**

**Table 1**. $^1$H-MRI contrast of the reporters $M_1 \sim M_8$ in the presence of FAC. [**Conditions:** $T_1$-weighted $^1$H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×128, 50×50mm$^2$. (**A**) Control, $M_1$, $M_3$, $M_5$, $M_7$ each (5μmol), FAC (2.5μmol), PBS (0.1M, pH=7.4, 1.5mL); $M_2$, $M_4$, $M_6$, $M_8$ each (5μmol), FAC (2.5μmol), Tris buffer (50 mM, pH=7.4, 1.5mL); (**B**) Complex, $M_1 \sim M_8$ each (5μmol), FAC (2.5μmol), E801A (10 units), PBS (0.1M, pH=7.4, 1.5mL)], or PSMA (from lysed LNCaP cells 5×10$^6$), Tris buffer (50 mM, pH=7.4, 1.5mL).

| Enzyme | β-galactosidase E801A | | | | PSMA | | | |
|---|---|---|---|---|---|---|---|---|
| Molecule | $M_1$ | $M_3$ | $M_5$ | $M_7$ | $M_2$ | $M_4$ | $M_6$ | $M_8$ |
| $T_1$-weighted $^1$H MRI (Control) |  |  |  |  |  |  |  |  |
| $T_1$-weighted $^1$H MRI (Enzyme) |  |  |  |  |  |  |  |  |

The molecular modeling shows that the fused structure of $Gd^{3+}$ and $Fe^{3+}$ chelator results in the molecular structures of $M_1 \sim M_8$ very rigid (*see* **Figure 10** as an example of $M_1$, $M_2$). Therefore, we deduced that these too rigid molecules would be hard to coordinate with PSMA or *lacZ* proteins and dock into the cleft of the active site of the enzymes for interaction.[112,113]
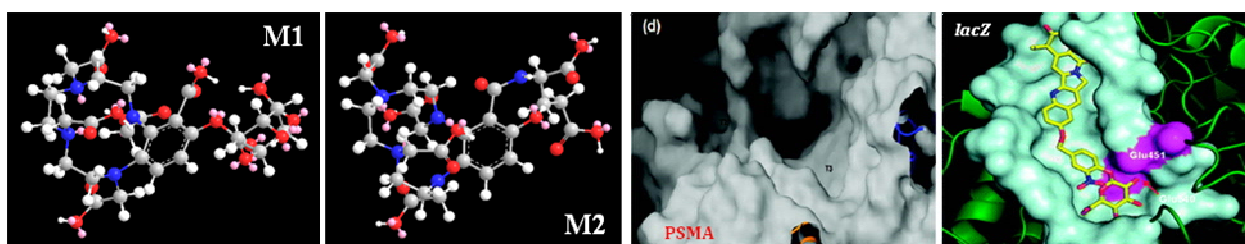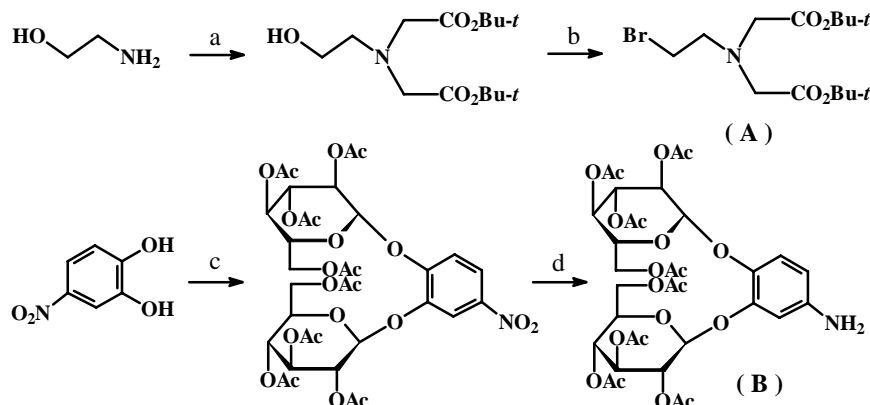


**Figure 10**

The molecular modeling and docking studies provide us a better understanding of the interactions between our designed target molecules and PSMA or *lacZ* proteins. We found that selecting a suitable structure with certain features (*e.g.* flexibility, linkage, appropriate angles, versatile binding modes, and coordination to $Fe^{3+}$ with plastic chelating geometry) is crucial to the construction of the enzyme responsive enhanced MRI contrast agents. We also realized that the previous design and synthesis involved too many steps of reactions, not briefly and efficiently, displaying the increasing difficulty either in synthesis and purification with lower yields, especially, when the molecules grew bigger.

With these considerations in mind, we tried to introduce diethylenetriamine-*N,N',N'',N''*-tetraacetate (DTTA) instead of the cyclic DOTA or PCTA as Gd(III) chelator (see **Figure 11**) with a straightforward strategy. Most importantly, molecule $M_9$ can be hydrolyzed by β-galactosidase E801A in the presence of FAC in PBS (0.1 M, pH=7.4), producing obvious MRI contrast change before and after reaction with β-galactosidase E801A (see **Figure 12**), implying we found the right way. Inspirited by these results, we extended this strategy further for the synthesis of other new molecules by using the Fe-chelation agents in anticancer therapy as the Fe-chelator for construction of the responsive MRI contrast agents. We firstly proved that the clinically applied Fe-chelators, such as **P**yridoxal **I**sonicotinoyl**H**ydrazone (**PIH**), and its analogues **S**alicylaldehyde **B**enzoyl**H**ydrazone (**SBH**), **S**alicylaldehyde **I**sonicotinoyl**H**ydrazone (**SIH**) and **S**alicylaldehyde **N**icotinoyl**H**ydrazone (**SNH**), can act as

Fe-based ${}^{1}$H MRI contrast agents to produce strong $T_1$-weighted contrast effects (**Figure 13**, TABLE 2), suggesting that the Fe-complex formation could not only localize, accumulate and restrict the motion of the linked Gd-based ${}^{1}$H MRI moiety, but also itself can produce the additional relaxivity.



**Reaction Conditions:** (a) BrCH$_2$CO$_2$Bu-$t$, KHCO$_3$, 88%; (b) Ph$_3$P, NBS, 86%; (c) 2,3,4,6-tetra-$O$-acetyl-$a$-D-galactopyranosyl bromide, Hg(CN)$_2$, 4Å M.S., CH$_2$Cl$_2$, 92%; (d) H$_2$, Pd/C, 100%.
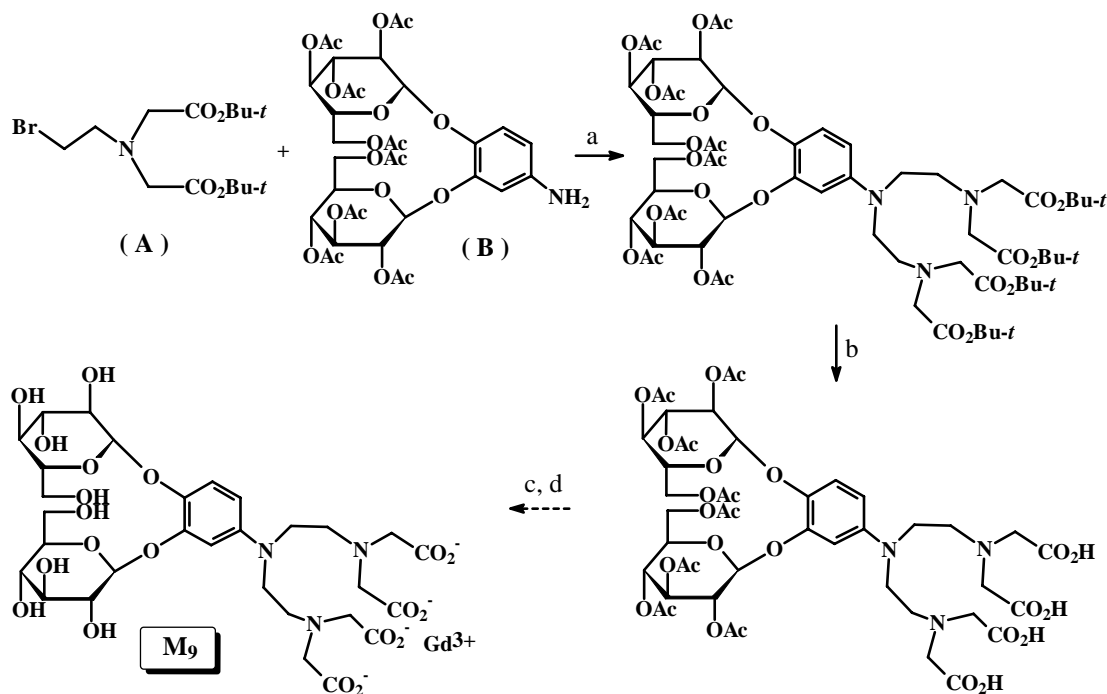


**Figure 11. Reaction Conditions:** (a) K$_2$CO$_3$, MeCN, 78%; (b) CF$_3$CO$_2$H, CH$_2$Cl$_2$, 84%; (c) GdCl$_3$, Pyridine, 80%; (d) MeOH, MeONa, 86%.
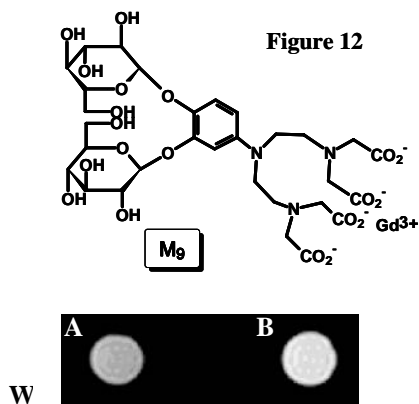


**Figure 12**. $T_1$-weighted (TR/TE 250/12 ms) MR images of solutions and the signal intensity in test tubes at 4.7 T MR scanner: (**A**) PBS with **M$_9$** and FAC; (**B**) PBS with **M$_9$**, FAC and β-galactosidase E801A.
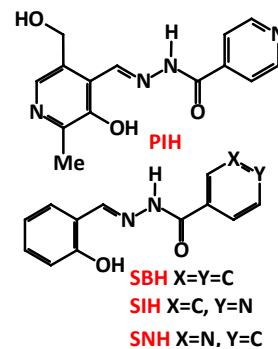
**Figure 13**

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Then, we synthesized $M_{10}$, $M_{11}$ and $M_{12}$ by using **SBH**, **SIH** and **SNH** as the Fe-chelators (see **Figure 14**). The MRI evaluation of the reporter molecules $M_{10}$, $M_{11}$ and $M_{12}$, respectively, in sodium phosphate buffer solution (PBS) (0.1 M, pH=7.4) in the presence of ferric ammonia citrate (FAC) with β-galactosidase E801A indicated that: (1) Again, the reporter molecule $M_{10}$, similarly like $M_9$, can be hydrolyzed by β-galactosidase E801A,

| Chelator | PIH | SBH | SIH | SNH |
|---|---|---|---|---|
| $T_1$-weighted $^1$H MRI (Control)[1] | | | | |
| $T_1$-weighted $^1$H MRI (Control)[2] | | | | |
| $T_1$-weighted $^1$H MRI (Complex) | | | | |

**TABLE 2.** (**A**) (1) Control, **PIH**, **SBH**, **SIH** or **SNH** each (1.6mM) in PBS; (2) Control, ferric ammonia citrate (FAC) (0.8mM) in PBS; (**B**) Complex, **PIH**, **SBH**, **SIH** or **SNH** each (1.6mM), FAC (0.8mM) in PBS; **CONDITIONS**: $T_1$-weighted $^1$H MRI, 200MHz, TR=300ms, TE =20ms, 1.5mm slice, 128×128, 50×50mm$^2$.

producing apparent MRI contrast change upon response to β-galactosidase E801A; (2) Unlike $M_9$ and $M_{10}$, $M_{11}$ and $M_{12}$ have no MRI contrast enhancements with galactosidase E801A in the presence of FAC in PBS (0.1 M, pH=7.4), but with strong MRI contrast changes with galactosidase G5160 in the presence of FAC in PBS (0.1 M) at pH=4.5 (**Table 3**).



( A )



( B )

**Reaction Conditions:** (a) BrCH$_2$CO$_2$Bu-$t$, KHCO$_3$, 88%; (b) Ph$_3$P, NBS, 86%; (c) PhCONHNH$_2$, EtOH, AcOH, Reflux, 82%; (d) H$_2$, Pd/C, 96%.



$M_{10}$ X = Y = C
$M_{11}$ X = C, Y = N
$M_{12}$ X = N, Y = C

**Figure 14. Reaction Conditions:** (a) K$_2$CO$_3$, MeCN, 42%; (b) CF$_3$CO$_2$H, CH$_2$Cl$_2$, 64%; (c) GdCl$_3$, Pyridine, 70%; (d) MeOH, MeONa, 85%.

**Table 3**. [1]H-MRI contrast of the reporters $M_{10} \sim M_{12}$ in the presence of FAC with E801A in PBS (0.1M, pH=7.4) or G5160 in PBS (0.1M, pH=4.5). [**Conditions:** $T_1$-weighted ^{1}H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×128, 50×50mm$^2$**. (A)** Control, $M_{10} \sim M_{12}$ each (5μmol), FAC (2.5μmol), PBS (0.1M, pH=7.4, 1.5mL); **(B)** Complex, $M_{10} \sim M_{12}$ each (5μmol), FAC (2.5μmol), E801A (10 units), PBS (0.1M, pH=7.4, 1.5mL)], or G5160 (10 units), PBS (0.1M, pH=4.5, 1.5mL)].

| Enzyme | β-galactosidase E801A | | | β-galactosidase G5160 | | |
|---|---|---|---|---|---|---|
| Molecule | $M_{10}$ | $M_{11}$ | $M_{12}$ | $M_{10}$ | $M_{11}$ | $M_{12}$ |
| $T_1$-weighted ^{1}H MRI (Control) | | | | | | |
| $T_1$-weighted ^{1}H MRI (Enzyme) | | | | | | |

The experience accumulated on the development of enzyme responsive enhanced MRI contrast agents opened our mind, and the desire for an ideal *in vivo* MRI probe prompted us to design and syntheses another two kinds MRI agents: (1) through phenylthioureido as linkage connecting $Gd^{3+}$ and $Fe^{3+}$ chelators for suitable flexibility of the molecules $M_{13}$ and $M_{14}$ (see **Figure 15**), both like $M_9$ produced apparent MRI contrast differences upon response to β-galactosidase E801A.



Figure 15
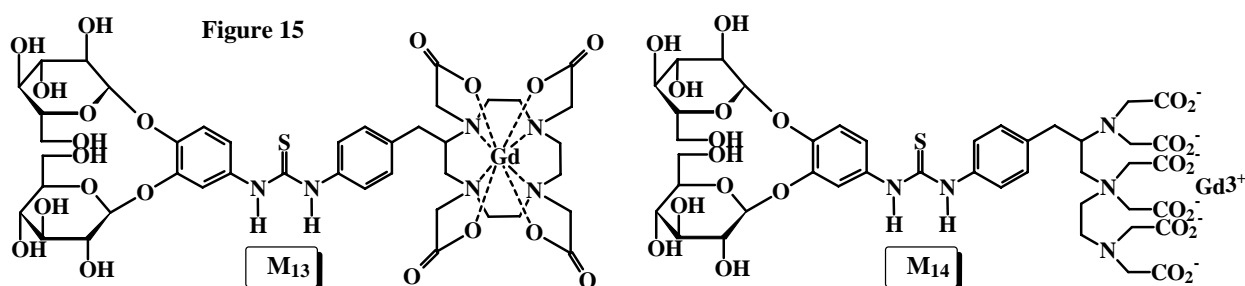
(2) "Click Chemistry" Approach   Because of regioselectivity, high yields in reasonable reaction times under mild conditions, "Click Chemistry" has been applied in a wide range of fields from synthetic chemistry to biomedicine and materials science. Our attention is on the versatile triazole rings as linkers between $Fe^{3+}$ and $Gd^{3+}$-ligands to functionalize with tolerance for the interaction with *lacZ* protein (see **Figure 16**).

*In Vitro* **MRI Studies   (1) Cell preparation** (**a**) Stably transfected PC3 cell line: *E. coli lacZ* gene (from pSV-β-gal vector, Promega, Madison,WI) was inserted into high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, San Diego, CA) giving a recombinant vector phCMV/*lacZ*, which was used to transfect PC3 cells using GenePORTER2 (Gene Therapy Systems). Cells were grown in DMEM (Dulbecco's Modification of Eagle's Medium, Mediatech, Inc, Herndon, VA), 10% FBS (Fetal bovine serum, Hyclone, Logan, UT) with 1% Penicillin-streptomycin Solution (Mediatech). The highest β-gal expressing colony was selected using G-418 disulfate ($C_{20}H_{40}N_4O_{10}$. $2H_2SO_4$, Research Products International Corp, Mt. Prospect, IL) (800 μg/ml), which was also included for routine culture (200 μg/ml). with 1% Penicillin-streptomycin Solution (Mediatech). The highest β-gal expressing colony was selected using G-418 disulfate

($C_{20}H_{40}N_4O_{10}$. $2H_2SO_4$, Research Products International Corp, Mt. Prospect, IL) (800 µg/ml), which was also included for routine culture (200 µg/ml).
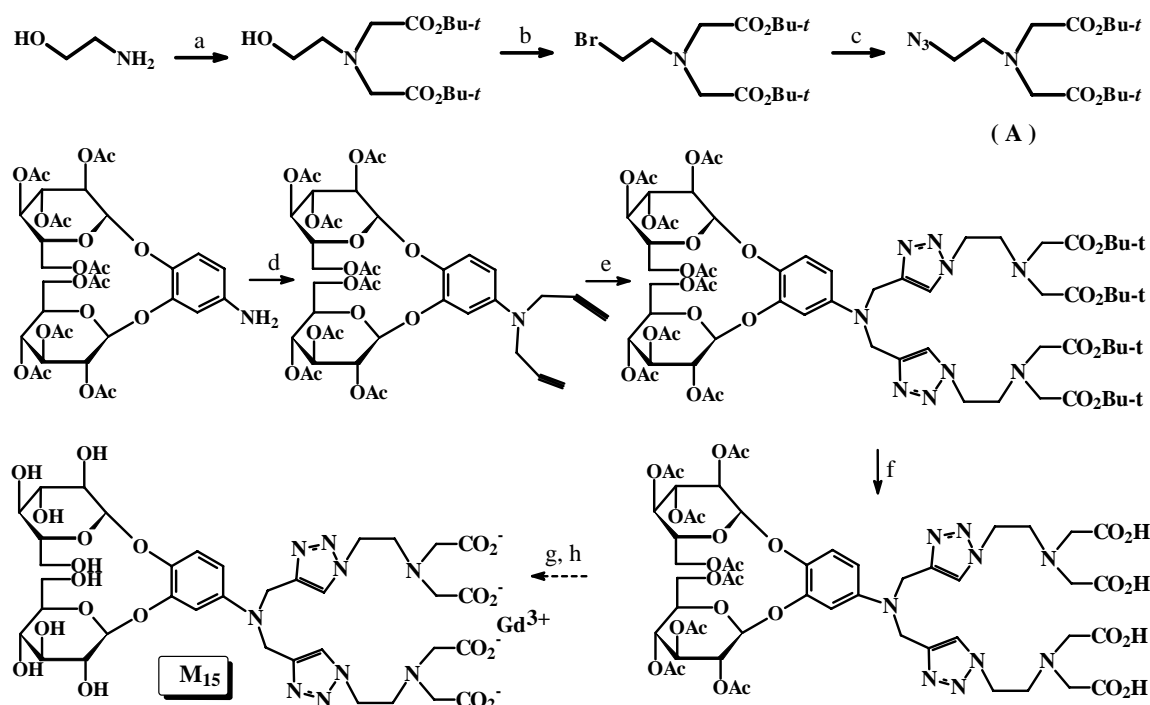


**Figure 16. Reaction Conditions:** (a) $BrCH_2CO_2Bu$-$t$, $KHCO_3$, 88%; (b) $Ph_3P$, NBS, 86%; (c) $NaN_3$, DMF, 80C, 88%, (d) $CHCCH_2Br$, $K_2CO_3$, DMF, rt, 86%, (e) (A), $CuSO_4$, NaAsc, t-BuOH, rt, 69%, (f) $CF_3CO_2H$, $CH_2Cl_2$, 74%; (g) $GdCl_3$, Pyridine, 71%; (h) MeOH, MeONa, 82%.

(**b**) X-gal and S-gal staining for β-gal: cells were fixed in PBS plus 0.5% glutaraldehyde (5 min) and rinsed in PBS prior to staining. Staining was performed using standard procedures for 2 hours at 37 °C in PBS plus 1 mg/ml X-gal (Sigma, St. Louis, MO), 1 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ or with 1.5 mg/ml S-gal (Sigma) and 2.5 mg/ml FAC (see **Figure 17**). (**c**) β-Gal activity assay: The β-gal activity of tumor cells and tissues in mice was measured using the β-gal assay kit (Promega, Madison, WI) with yellow *o-*
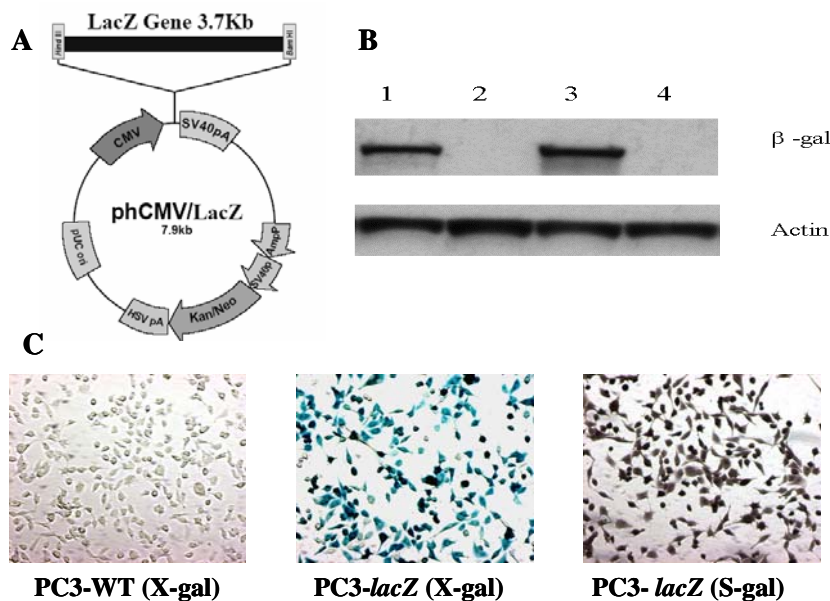


**Figure 17** Generation of PC3 cells stably expressing of β-gal. (**A**) Map of recombinant *lacZ* vector (phCMV/*lacZ*). (**B**) Western blot: cell extracts of two transfected lines PC3-*lacZ*1 (lane 1) and PC3-*lacZ* (lane 3), together with PC3-WT (lanes 2 and 4) were examined. (**C**) PC3 wild-type and PC3-*lacZ* cells were stained using X-gal and S-gal: over 90% of PC3-*lacZ* cells were stained blue or black, respectively, while the PC3 wild type cells did not stain.

nitrophenyl β-*D*-galactopyranoside. (**d**) Western blot analysis: Protein was extracted from PC3 tumor cells and was quantified by a protein assay (Bio-Rad, Hercules, CA) based on the Bradford method. Each well was loaded with 30μg protein and separated by 10% SDS-PAGE (Nu-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Primary monoclonal anti-*β*-gal antibody (Promega) and anti-actin antibody (Sigma) were used as probes at a dilution of 1:5000, and reacting protein was detected using a horseradish peroxidase-conjugated secondary antibody and ECL detection (Amersham, Piscataway, NJ).

(**2**) *In Vitro* **MRI**    The *in vitro* evaluation of **M$_9$**~**M$_{14}$** with PC3-*lacZ* cells in the presence of FAC showed that only molecules **M$_9$** and **M$_{10}$** exhibited apparent MRI differences.    **M$_9$** and **M$_{10}$** (6 μmol) each in 1:1 DMSO/PBS was added to suspensions of $5\times10^6$ PC3 wild type and PC3-*lacZ* cells in PBS (1.0 mL) and FAC (3 μmmol) in wells and maintained at 37$^o$C. MRI experiments were performed on a 4.7 T Varian Unity INOVA spectrometer. **Figure 18** showed the *in vitro* MR images of **M$_9$** and **M$_{10}$**
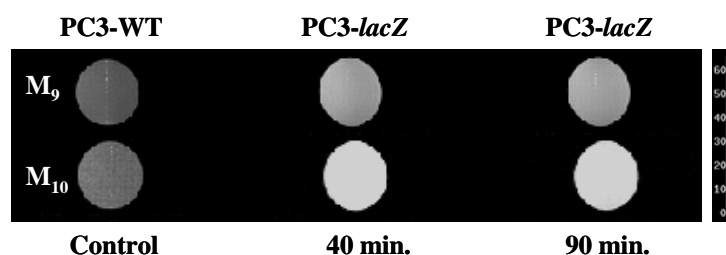


**Figure 18** $^1$H MRI, 200 MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×64, 40×40 mm$^2$. (**A**) control, **M$_9$** or **M$_{10}$** (6 μmol), FAC (3 μmol), $5\times10^6$ PC3 WT, PBS (0.9 mL), DMSO (0.1 mL); (**B**) **M$_9$** or **M$_{10}$** (6 μmol), FAC (3 μmol), $5\times10^6$ PC3-*lacZ*, PBS (0.9 mL), DMSO (0.1 mL)].

with *lacZ* transfected prostate tumor cells, yielding obvious MRI contrast changes between in WT and *lacZ* transfected PC3 prostate tumor cells, indicating that both **M$_9$** and **M$_{10}$** can penetrate prostate tumor PC3 cell membrane and have no apparent cytotoxicity and no physiological perturbation effects on WT and *lacZ* transfected PC3 cells, the others **M$_{11}$**~**M$_{14}$** cannot cross prostate tumor PC3 cell membrane.

*In Vivo* **MRI Studies of M$_9$ and M$_{10}$**  (**1**) **Animal model**    All *in vivo* MRI studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC). Wild type and stably transfected *lacZ* PC3 cells ($2\times10^6$) were implanted subcutaneously in the left and right thighs of mice, respectively, when the tumors reached ~0.8 cm in diameter, the mouse was anesthetized (1.3% isoflurane/air at 1 dm$^3$/min) with a facemask and maintained at 37°C by a warm pad with circulating water, and placed into animal coil for imaging. MRI data were acquired using a 4.7 T horizontal bore magnet with a Varian INOVA Unity system (Palo Alto, CA, USA). $T_1$ and $T_2$ values were measured using a spin echo sequence with varying repetition and echo times, *e.g.* $T_1$-weighted $^1$H MRI, 200MHz, TR=0.2, 0.3, 0.5, 0.8, 1, 1.5, 2, 4, 6s,



**PC3-*lacZ* Tumor (X-gal)**

**PC3-WT Tumor (X-gal)**

**Figure 19**

TE=12ms, 1.5mm slice, matrix=128×128, FOV=50×50mm$^2$; $T_2$-weighted $^1$H MRI, 200MHz, TR=6s, TE=11, 15, 20, 30, 50, 100, 150ms, 1.5mm slice, matrix=128×128, FOV=50×50mm$^2$. Histology analysis confirmed that PC3-*lacZ* tumor section showed over 90% of tissue stained blue for β-gal, while PC3-WT tumor histological section showed little or no blue stain (**Figure 19**). (**2**) *In Vivo* **MRI with i.v. injection** Mice bearing PC3-WT and PC3-*lacZ* tumors were imaged on a 4.7 T Varian Unity INOVA spectrometer. T1-weighted transaxial images were obtained before and after intravenous injection of the
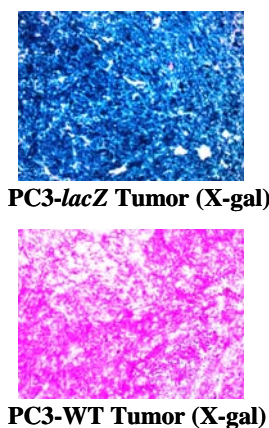
mixture of 0.4 mmol/kg $M_9$ and $M_{10}$ and FAC. Postcontrast scans were obtained every 15 min for one and half hours. For both reporters $M_9$ and $M_{10}$, the MR images of animals showed that there are no time-signal intensity changes between PC3-WT and PC3-*lacZ* tumors before and after $M_9$ and $M_{10}$ injection (**Figure 20**), indicating that both $M_9$ and $M_{10}$ can either be washed out or metabolized very quickly, and can't reach to PC3-WT and PC3-*lacZ* tumors on the thighs with enough amount. Also, we found that mice all died one and half-hours later after intravenous injection of $M_9$. **(3) *In Vivo* MRI with direct injection into tumors** However, if a solution of $M_{10}$ (0.4 mmol/kg) and FAC (DMSO/PBS 1:1 V/V') was injected directly into the tumors



**Control**          **40 min**          **90 min**

**Figure 20**   $^1$H MRI, 200 MHz, TR=250ms, TE=12ms, 1.5mm slice, 128×64, 40×40 mm$^2$. (**A**) control; (**B**) $M_{10}$ (0.4 mmol/kg), FAC (0.2 mmol/kg), PBS/DMSO (1:1), i.v. injection].



**Control**          **25 min**          **45 min**          **75 min**

**Figure 21**  $^1$H MRI, 200 MHz, TR=250ms, TE=12ms, 1.5mm slice, 128×64, 40×40 mm$^2$. (**A**) control; (**B**) $M_{10}$ (0.4 mmol/kg), FAC (0.2 mmol/kg), PBS/DMSO (1:1), i.v. injection].

in a "fan" pattern, strong contrast was detected in the *lacZ* expressing PC3 tumors (**Figure 21**).

### RESEARCH ACCOMPLISHMENTS

(1) Designed and synthesized a series of reporter molecules, and verified their structures, importantly, accumulated solid foundation, experience and expertise for the further investigation on molecular imaging.

(2) Finished the *in vitro* and *in vivo* evaluation of the reporter molecules $M_9$ and $M_{10}$, and the results demonstrated this novel mechanism proposed in W81XWH-05-1-0593 for imaging and evaluation of prostate cancer gene therapy is reliable.

### REPORTABLE OUTCOMES

(1) A series of abstracts had been accepted for presentation on the various conferences such as World Molecular Imaging Congress, *Innovative Minds in Prostate Cancer Today*, American Chemical Society Meeting.

(2) Several papers are in preparation.

### CONCLUSIONS

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States. The advent of effective screening measures can sharply decrease the mortality of prostate cancer through detecting this disease at an earlier stage. However, the evidence for mortality benefit from prostate cancer screening has been disappointing to date. Expanding knowledge of prostate cancer biology with combination of imaging technologies would be of considerable value in many ongoing and future clinical prostate cancer diagnosis and gene therapy trials.

Based on the biologic features of prostate cancer, we proposed in this project a new approach for *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging. The ultimate objective is to demonstrate the utility and reliability of this new approach to measure β-gal or PSMA activities *in vivo*. We have accomplished a series of target molecules **M₁~M₁₄,** and verified by NMR data. Strong MRI contrast changes of target molecules **M₉** and **M₁₀** for detection *lacZ in vitro* and *in vivo* demonstrated this novel mechanism described in W81XWH-05-1-0593 is feasible and reliable. With screening out the ideal reporter molecules, we believe the translation of this novel approach to clinical investigations will enable prostate cancer detection comprehensive and infallible.

**Prostate Cancer Evaluation: Design, Synthesis and Evaluation of Novel Enzyme-Activated $^1$H MRI Contrast Agents**

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**APPENDICES**

(1) Jian-Xin Yu, R. P. Mason, Design and Synthesis of Novel *lacZ* Responsive Enhanced MRI Agent, *The World Molecular Imaging Conference*, #0614, Montreal, Canada, September 23-26, 2009.

(2) Jian-Xin Yu, Dawen Zhao, R. P. Mason, Design and Synthesis of Novel *lacZ* Responsive Enhanced MRI Agent, *The World Molecular Imaging Conference*, #0615, Montreal, Canada, September 23-26, 2009.

Figure: Development of Novel Fe-Based $^1$H MRI *lacZ* Gene Reporters for *in vivo* Assessment — Jian-Xin Yu, Dawen Zhao, Ralph P. Mason, Cancer Imaging Program, Department of Radiology, University of Texas Southwestern Medical Center at Dallas, Texas, USA. (Presentation: 0614)

DEVELOPMENT OF NOVEL *lacZ* RESPONSIVE ENHANCED Gd-BASED MRI AGENT

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Presentation: 0615

### INTRODUCTION

Gene therapy holds great promise for treating cancer and has been successfully exploited in several clinical trials. A major current obstacle to implementation is to establish a method of assessment of therapeutic gene expression in terms of heterogeneity and longevity in tissues. The *lacZ* gene encoding β-galactosidase (β-gal) has been recognized as the most attractive reporter gene. Therefore, noninvasive detection *in vivo* would be of considerable value in future clinical cancer gene therapy trials.[1] various groups have presented innovative approaches to assessing β-gal activity *in vivo* using gadolinium enhanced contrast ^{1}H MRI or ^{19}F NMR spectroscopy,[2-6] optical[7-11] and radionuclide imaging.[12-14] We now report the exploration of a novel approach to *lacZ* responsive Gd-based ^{1}H MRI agents.

Desreux *et al* demonstrated that, by chelating Gd(phen)HDO3A with Fe(II) to form a highly stable tris-complex, the relaxivity increased 145% at 20MHz and 37°C.[15] Most recently, Tóth *et al* showed that the heterometallic, self-assembled metallostar with six efficiently relaxing GdIII centers, $[Fe\{Gd_2L(H_2O)_2\}_3]^{4-}$, exhibited a particularly high relaxivity for its moderate molecular weight.[16]

Iron is a critically important metal ion for a wide variety of cellular events. Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of more iron. Many studies indicate that a high level of iron accumulation in animals and humans is associated with both the initiation and the progression of cancers. Cancer cells are also sensitive to iron depletion because of the critical requirement for iron in proteins that play essential roles in DNA synthesis, repair and energy production. Iron depletion as a therapeutic strategy has been applied for cancers. The FDA has already approved five iron chelators for use in anticancer therapy; others are being evaluated in clinical settings.[17]

### DESIGN

Prompted by this finding, we now propose a novel class of enzyme activated Gd3+-based MRI contrast agent for *in vivo* detection of β-gal activity combing three features: (A) a signal enhancement group; (B) an Fe3+ chelating group using the structure of clinical iron chelation therapy drugs; (C) β-*D*-galactose. Following cleavage by β-gal in cells, the released, activated aglycone Fe3+-ligand will spontaneously trap endogenous Fe3+ at the site of enzyme activity forming a highly stable complex, exhibiting restricted motion of the Gd3+ chelates enhancing relaxivity and providing contrast based on gene (*viz.* enzyme) stimulated local accumulation (Figure 1).
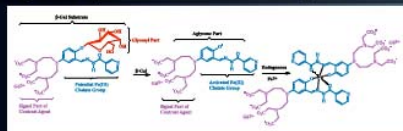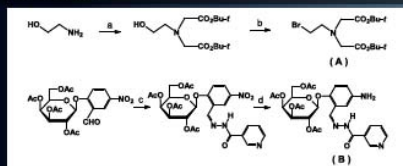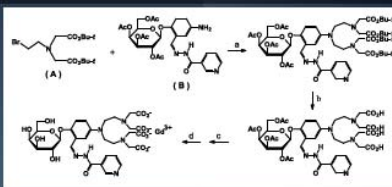


Figure 1. The proposed new mechanism for *in vivo* detection of *lacZ* gene expression through Fe3+-trapped MRI contrast agent formation.
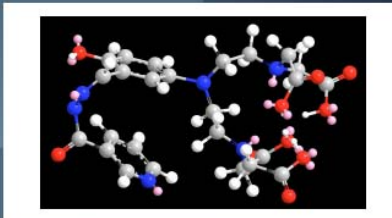
### SYNTHESIS



Reaction Conditions: (a) BrCH2CO2Bu-t, KHCO3, 88%; (b) Ph3P, NBS, 86%; (c) Nicotinic Hydrazide, EtOH, AcOH, Reflux, 85%; (d) H2, Pd/C, 94%.
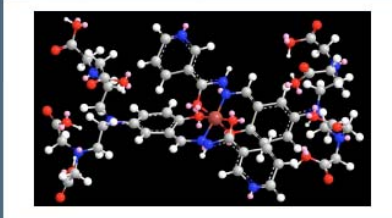
### SYNTHESIS



Reaction Conditions: (a) K2CO3, MeCN, 38%; (b) CF3CO2H, CH2Cl2, 52%; (c) GdCl3, Pyridine, 57%; (d) MeOH, MeONa, 75%.
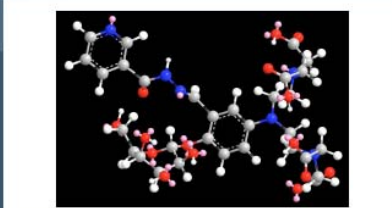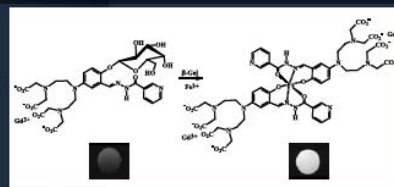
### STRUCTURAL FEATURES



Ligand Structure



Fe-Complex Structure



Conjugate Structure

### RESULTS



Conditions: T1-weighted 1H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128-128, 50-50mm2. (A) Control, sample (5μmol), FAC (2.5μmol), PBS (0.1M, pH=4.5, 1.5mL); (B) Complex, sample (5μmol), FAC (2.5μmol), G-5160 (5 units), PBS (0.1M, pH=4.5, 1.5mL).

### CONCLUSIONS

We propose a novel class of enzyme activated Gd3+-based MRI contrast agent for detection of β-gal activity. Synthesis of the substrate has been successful, and the evaluation of this agent with β-gal showed that the released aglycone including the activated Fe3+-ligand and MRI signal enhancement group spontaneously traps Fe3+ in the solution forming a highly stable complex, then restricting the motion of the Gd3+ chelates enhancing relaxivity. We are now evaluating this agent with β-gal expressing cells and *in vivo*.

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### ADDITIONAL INFORMATION

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